# HIV-1 and Morphine Regulation of Autophagy in Microglia: Limited Interactions in the Context of HIV-1 Infection and Opioid Abuse

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## ABSTRACT

**Microglia are the predominant resident central nervous system (CNS) cell type productively infected by HIV-1, and play a key role in the progression of HIV-associated dementia (HAD). Moreover, neural dysfunction and progression to HAD are accelerated in opiate drug abusers. In the present study, we examined the role of the autophagy pathway in the neuropathogenesis of HIV-1 using primary human microglial cells and determined whether opiates converge at this point. Infection of microglia with the HIV-1$_{SF162}$ macrophage-tropic strain resulted in increased Beclin1 expression, accompanied by an increase of LC3 protein levels and accumulation of LC3 reporter RFP$^+$ GFP$^+$ (yellow) puncta, suggesting that HIV-1 infection triggers autophagosome formation without promoting protein degradation by the lysosome. Conversely, coexposure with HIV-1 and morphine significantly decreased virus-induced Beclin1 expression and autophagosome formation. Exploration of the possible mechanism(s) used by morphine to disrupt the autophagic process unveiled a significant increase in intracellular pH, which coincided with a reduction in the formation of acidic vesicular organelles and in autophagolysosome formation. Small interfering RNA targeting BECN1, a gene critical for autophagosome formation, significantly reduced viral replication and the virus-induced inflammatory responses. Conversely, morphine-enhanced viral replication and inflammatory responses were not affected by gene silencing with siBeclin1, suggesting that the interactive effect of morphine in HIV-1 pathogenesis is mediated through a Beclin1-independent mechanism. These novel findings may have important implications on the connections between autophagy and HIV-1 pathogenesis mediated by microglial cells in opioid-abusing individuals.**

## IMPORTANCE

**About 50% of individuals infected with HIV-1 will develop some sort of neurocognitive impairment that cannot be prevented nor eradicated by antiretroviral therapy. The neuropathogenesis is mostly due to inflammatory responses by infected microglia, the resident immune cells of the brain. Cognitive disorders may also be associated with drugs of abuse. In fact, opioid drug users have an increased risk of developing neurocognitive disorders with increased progression to dementia. Although the mechanism(s) by which opioids exacerbate the neuropathogenesis of HIV-1 are not entirely known, it is well accepted that glia are critical to opiate responses. This study gives us new insight into possible autophagic mechanism(s) in microglia that control HIV-1 replication and virus-induced inflammation in the context of opioid abuse and should greatly improve our knowledge in the pathogenesis of HIV-1 resulting from substance abuse to provide a better understanding for the design of candidate antiviral therapies targeting drug-abusing individuals.**

Microglia are macrophage-like resident immune cells in the brain that can be activated by HIV-1 infection itself, by interaction with exogenous viral proteins, or in response to various cellular factors, including cytokines, chemokines, nitric oxide (NO), and reactive oxygen intermediates secreted from infected cells (1). Prolonged or excessive activation of microglia produces inflammatory reactions in the brain, which are believed to be the primary cause of neuronal injury or dysfunction related to HIV-1-associated dementia (HAD) pathology (2). Therefore, the signaling pathways leading to macrophage or microglial activation are potential therapeutic targets for the prevention or treatment of HAD. Opiate drug abuse and HIV-1 are interlinked epidemics (3, 4), and opiates such as heroin can exacerbate the neuropathogenesis of HIV-1 with a swift progression to neuroAIDS (5). Although the mechanism(s) by which opioids exacerbate the neuropathogenesis of HIV-1 are not entirely known, it is well accepted that glia are critical to opiate responses (5–7). In the central nervous system, heroin is quickly deacetylated to morphine, which is the

main bioactive product of heroin in the brain (8, 9). Morphine was therefore the compound that was tested in the present study.

Macroautophagy (here "autophagy") is a multistep catabolic process that is regulated by numerous different autophagy-related (ATG) genes and consists of several stages through which cytoplasmic material, including long-lived proteins, aggregated pro-

teins and dysfunctional organelles, are delivered to the lysosome for degradation (10, 11). Autophagy also plays a central role in the innate and adaptive immunity of many types of immune cells, including macrophages, with diverse functions such as the regulation of inflammatory responses, antigen presentation, and pathogen elimination (12–15). Autophagy can be regulated in response to immune stimuli, including cytokines, and autophagy itself can regulate the production and secretion of these factors (16, 17). Autophagy can also be induced by various environmental and cellular stresses, such as nutrient starvation and trophic factor withdrawal (11). The autophagy pathway is under the control of two key kinases and nutrient sensors, namely, the mammalian target of rapamycin (mTOR), a Ser/Thr kinase which inhibits autophagy, and AMP-activated protein kinase, a positive regulator of autophagy (18). Dysregulation of autophagy has been associated with a variety of pathological conditions, including cancer, as well as cardiovascular, pulmonary, and neurodegenerative diseases (19–22).

Numerous studies have shown that autophagy influences the viral replication cycle and silencing of autophagic factors results in the long-term inhibition of HIV-1 replication (23–25). In addition, morphine increases the infection of monocytes with CXCR4- and CCR5-tropic HIV-1 strains (26). However, the role of autophagy in HIV-1-infected microglia and its effect on viral replication and the release of virus-induced proinflammatory factors are largely unknown. Moreover, information regarding the impact of autophagy on the actions of drugs of abuse on glial cell function in the context of HIV-1 infection is very limited. Therefore, in the present study, the relationship between autophagy, HIV-1 infection, and morphine exposure in microglial cell function was addressed. Taken together, our data demonstrate that activation of the host autophagic pathway by HIV-1 infection represents an essential mechanism in controlling viral replication and virus-induced inflammatory responses in microglia, while morphine-enhanced viral replication and virus-induced inflammation are mediated through a Beclin1-independent mechanism. Targeting of host cellular cofactors by inhibiting autophagy and its regulatory components could be of therapeutic use (27, 28) in the management of HIV-associated neurocognitive disorders with implications for opioid-abusing individuals.

## MATERIALS AND METHODS

**Reagents and drug treatments.** Acridine orange (AO) was purchased from Sigma (St. Louis, MO). Small interfering RNA (siRNA) against Beclin1 and control siRNA were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Morphine sulfate was obtained from The National Institute on Drug Abuse (NIDA; Bethesda, MD) and used at a concentration of 500 nM. HIV-1 Tat$_{1-86\ IIIB}$ and gp120$_{ADA}$ were purchased from ImmunoDX, LLC (Woburn, MA), and used at a concentration of 100 nM and 500 pmol, respectively. The concentrations chosen for Tat and gp120 are of clinical relevance considering that many HIV patients on antiretroviral therapy have protein levels in plasma and/or tissues that are within these concentration ranges (29–31). Most importantly, Tat at a concentration of 100 nM and 500 pmol gp120 elicit functional deficits in glia and neurons similar to those occurring in HIV infection, and are considered to reflect levels seen pathophysiologically (6, 7, 32, 33).

**Transfection and HIV-1 infection of human microglia.** Primary human microglia (ScienCell, Carlsbad, CA) cultured in Microglia Medium (MM; catalog no. 1901; ScienCell) were grown in 24-well glass bottom culture plates (MetTek Corp., Ashland, MA) to ~75 to 80% confluence and transfected with control siRNA, Beclin1 siRNA, or a tandem fluorescent (tfl)-mRFP-GFP-LC3 reporter construct (Addgene, Cambridge,

MA) in Opti-MEM using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. After 4 to 6 h, the transfection medium was replaced with MM, and at 48 h posttransfection the cells were infected with the HIV-1$_{SF162}$ strain (34), originally isolated by Jay A. Levy, at a concentration of 50 pg of HIV-1 p24/10$^6$ cells (35), in the presence or absence of morphine. At 24 h posttreatment, (tfl)-mRFP-GFP-LC3 transfected cells were fixed in 3.7% paraformaldehyde, followed by observing green and red punctum formation using a Zeiss LSM 700 laser scanning confocal microscope at ×63 magnification (Zeiss, Germany).

**ELISA.** Cell culture supernatants (precleared by brief centrifugation) were used to measure the levels of interleukin-1 (IL-1), IL-6, and IL-8, monocyte chemotactic protein 1 (MCP-1), regulated on activation, normal T cell expressed and secreted (RANTES), and tumor necrosis factor alpha (TNF-α) by enzyme-linked immunosorbent assay (ELISA; R&D Systems, Minneapolis, MN) according to the manufacturer's instructions. Culture supernatants containing HIV-1 particles were used to measure p24 protein levels by ELISA according to the manufacturer's protocol (Advanced Bioscience Laboratories, Rockville, MD). The optical density was read at $A_{450}$ on a Pherastar FS microtiter plate reader (BMG LabTech, Cary, NC).

**Immunoblotting.** Whole-cell lysates were prepared in radioimmuno-precipitation assay buffer supplemented with a mixture of protease and phosphatase inhibitors and analyzed by Western blotting. Primary antibodies against Beclin1 (1:500), ATG5 (1:500), LC3B (1:1,000), and p62/SQSTM1 (1:1,000) were from Novus Biologicals (Littleton, CO), and β-actin antibody (1:1,000) was from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Primary antibodies were monitored by incubation with goat anti-mouse- and goat anti-rabbit-conjugated secondary antibodies (Southern Biotech, Birmingham, AL). The immunoblots were exposed to SuperSignal West Femto substrate (Thermo Scientific, Waltham, MA) and visualized using a Kodak 440 imaging system (Kodak, Rochester, NY).

**Lysosomal content assay.** Cells were stained with 25 μg/ml AO for 15 min, washed in phosphate-buffered saline (PBS), and fixed using 3.7% paraformaldehyde, followed by examination under a Zeiss (AxioVision) fluorescence microscope (Zeiss, Germany) using a ×63 objective. Acidic vesicles were also quantified by flow cytometry after the cell pellet was stained with AO for 15 to 20 min, followed by washing the cells with PBS and resuspension in 500 μl of PBS. Green (510 to 530 nm) and red (650 nm) fluorescence emissions from 2,500 cells illuminated with blue (488 nm) excitation light were measured using a FACSCanto II flow cytometer (BD Biosciences, Franklin Lakes, NJ). The red/green fluorescence ratio for individual cells was calculated using FlowJo software (Ashland, OR).

**Lysosomal pH assay.** Intracellular pH was measured using the fluorogenic pH indicator dye pHrodo Red AM intracellular pH (pHi) indicator (Invitrogen, Carlsbad, CA) that quantifies cytosolic pH in the range of 9 to 4 with a pKa of ~6.5. An excitation/emission of 560/580 nm was read using a Pherastar FS microtiter plate reader (BMG LabTech). Microglia were loaded with pHrodo according to the manufacturer's protocol, followed by treatment with HIV-1 Tat and gp120 proteins, and morphine alone or in combination. After 8 and 24 h, the intracellular pH levels were measured. Absolute pH values were calculated by converting the arbitrary pH ratios of light excited at 560 nm and emitted at 580 nm using an intracellular pH calibration curve kit (Invitrogen).

**Cell viability assay.** Cell viability in microglial cultures was assessed after 48 h in cells transfected with control siRNA and Beclin1 siRNA by propidium iodide staining (Molecular Probes, Eugene, OR). Viable cells were quantified by flow cytometry using a FACSCanto II flow cytometer (BD Biosciences).

**Statistical analysis.** Data were analyzed using analysis of variance (ANOVA) techniques, followed by Bonferonni's *post hoc* test for multiple comparisons (GraphPad Software, Inc., La Jolla, CA). An alpha level of $P < 0.05$ was considered significant.
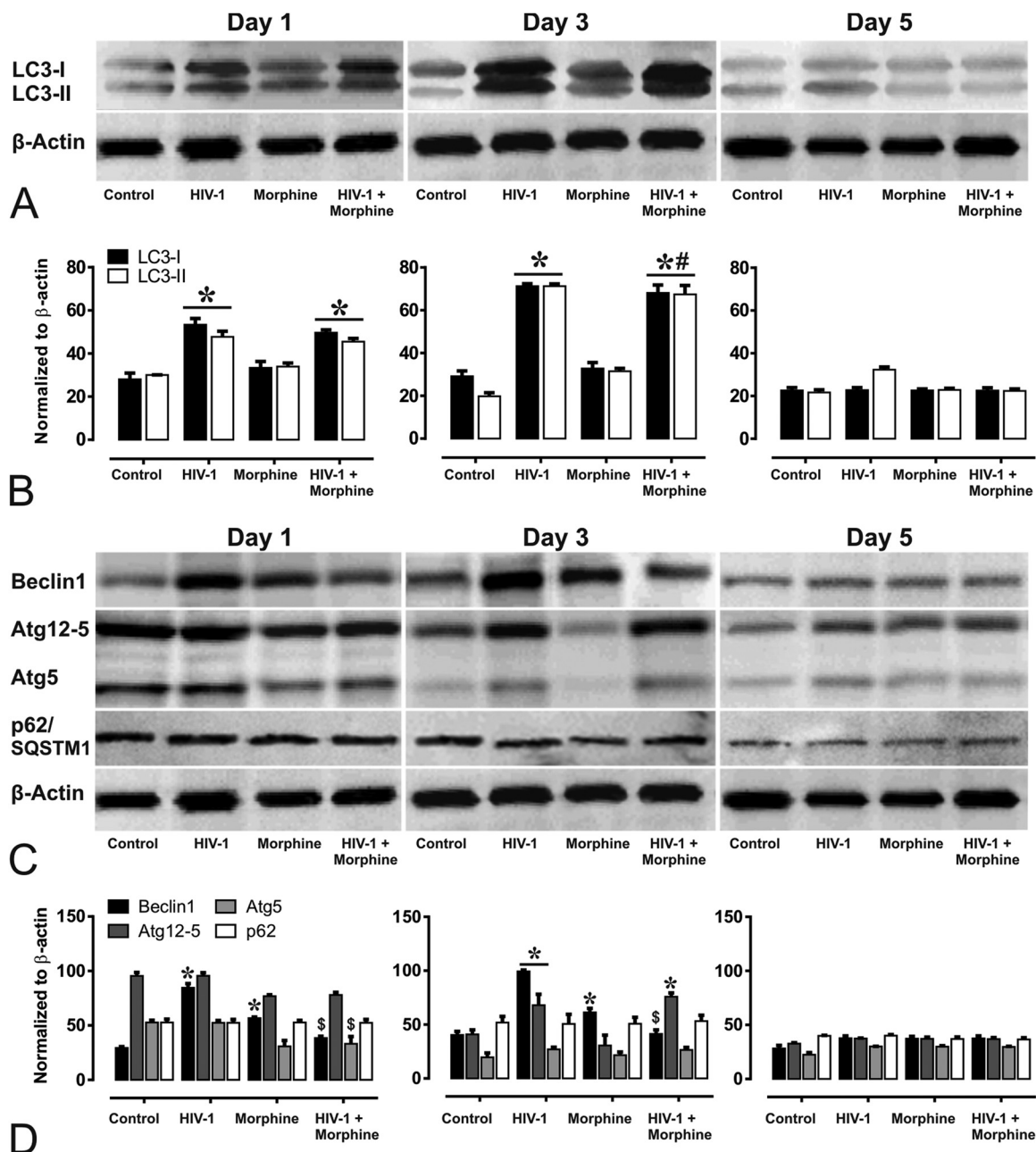
**FIG 1** HIV-1 infection and morphine induce the autophagic pathway in microglia. Human microglial cells were left untreated or infected with HIV-1 with or without morphine for 24 h, 3 days, or 5 days as indicated. Cell lysates were subjected to immunoblotting with antibodies to (A and B) LC3-I/II and (C and D) Beclin1, ATG12-5 conjugate, ATG5, and p62/SQSTM1. β-Actin was used as a loading control. Densitometry was performed for quantification, and the ratios of each protein to β-actin multiplied by 100 are presented graphically below their respective blots. Error bars show the standard errors of the mean (SEM) of three independent experiments. One-way ANOVA, followed by Bonferroni's *post hoc* test for multiple comparisons, was used to assess statistical differences (*, $P < 0.05$ versus control; $, $P < 0.05$ versus HIV-1; #, $P < 0.05$ versus morphine).

## RESULTS

**HIV-1 infection induces the autophagic pathway and shows limited interactions with morphine in microglia.** To begin to explore the connections between autophagy, HIV-1, and morphine exposure, an antibody that detects both LC3-I and LC3-II was used in Western blotting techniques to monitor autophagy during HIV-1 infection and morphine treatment of microglial cells. LC3 exists in two forms: LC3-I is present in a free cytoplas-mic form with a molecular mass of ~18 kDa, which upon lipidation with phosphatidylethanolamine (through a ubiquitin-like conjugation reaction) becomes associated with the membranes of autophagosomes and produces LC3-II, a useful marker of autophagic membranes with a molecular mass of ~16 kDa.

When the overall levels of LC3-I and LC3-II were normalized to β-actin, the expression of LC3-I and its conversion to the lipid bound form LC3-II significantly increased at days 1 and 3 postin-
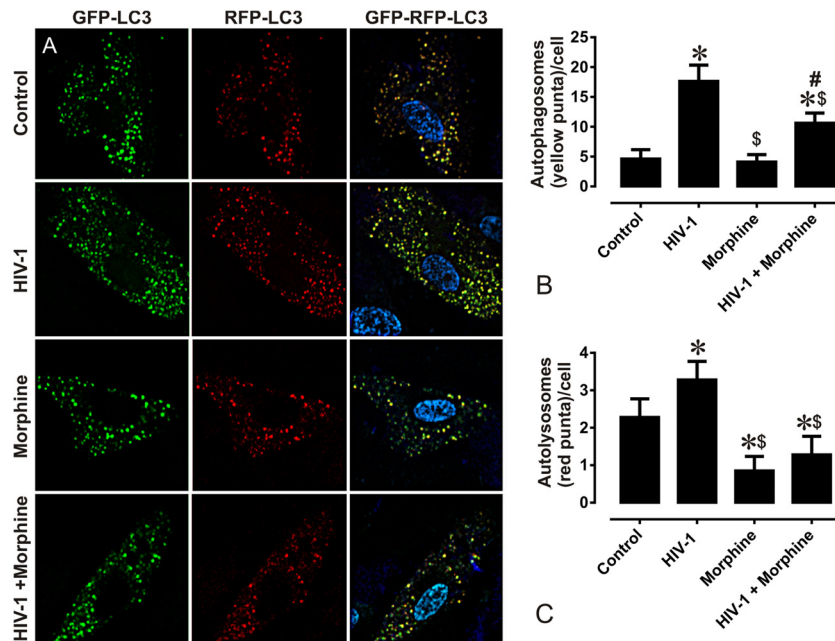
**FIG 2** Accumulation of autophagosomes with HIV-1 infection is decreased by morphine in microglia. Human microglia transfected with the mRFP-GFP-LC3 reporter were left untreated or infected with HIV-1 with or without morphine for 24 h. (A) Representative cells were examined for fluorescence using confocal microscopy at ×63 magnification. Colocalization of GFP and RFP indicated by yellow dots in overlapped GFP and RFP images is observed on autophagosomes, whereas only RFP fluorescence indicated by red puncta is observed on autolysosomes. Cells were stained with DAPI (blue) to label cell nuclei. (B) Quantification of autophagosomes (yellow; $GFP^+$ $RFP^+$ LC3) and autolysosomes (red; $GFP^-$ $RFP^+$ LC3) per cell from panel A. The data are presented as the average number of puncta per cell (± the SEM from three independent experiments) from 100 cells per experiment. One-way ANOVA, followed by Bonferroni's *post hoc* test for multiple comparisons, was used to assess statistical differences (*, $P < 0.05$ versus control; $, $P < 0.05$ versus HIV-1).

fection with HIV-1 (Fig. 1A and B). However, exposure to morphine treatment alone showed basal levels of LC3-I and LC3-II protein expression at days 1, 3, and 5, while concurrent exposure with HIV-1 infection had a minimal interactive effect compared to LC3 levels detected with HIV-1 infection alone (Fig. 1A and B).

In addition to LC3, the protein levels of Beclin1 and ATG12–5 were examined by Western blotting techniques to monitor autophagy. Beclin1 is a key component of the class III phosphatidylinositide 3-kinase complex, which is involved in the initiation of autophagosome formation (18, 36). The conjugation of ATG12 to ATG5 acts as an E3-like enzyme which is important for lipidation of the LC3 family of proteins and their association with vesicle membranes (37). When the levels of Beclin1 were normalized to β-actin, a marked increase in the protein expression levels of Beclin1 after days 1 and 3 postinfection was observed compared to control-treated cells (Fig. 1C and D). In addition, exposure to morphine alone caused an increase in the protein expression levels of Beclin1 after days 1 and 3 compared to control-treated cells, and caused a significant decrease in Beclin1 levels when in combination with HIV-1 infection (Fig. 1C and D). An antibody that detects both the ATG12-5 conjugate and ATG5 protein levels was then used and showed increased expression after days 1 and 3 postinfection with HIV-1 compared to control-treated cells. Exposure with morphine alone showed basal levels of both the ATG12-5 conjugate and ATG5 expression compared to control-treated cells, while in combination with HIV-1 infection there was a significant trend toward decreased HIV-1-induced protein expression at days 1 and 3 posttreatment (Fig. 1C and D).

We next examined p62/SQSTM1, which is a ubiquitin-binding scaffold protein that is selectively incorporated into autophago-

somes through direct binding to LC3 (38). Accordingly, both p62/SQSTM1 and LC3-II are degraded with ubiquitinylated proteins after autophagosome fusion with the lysosome, making p62/SQSTM1 a useful marker to study autophagic flux (38). The levels of p62/SQSTM1 were normalized to β-actin and showed a steady state of protein expression detected after days 1, 3, and 5 postinfection with HIV-1 that was not significantly different compared to control-treated cells (Fig. 1C and D). Likewise, exposure to morphine alone or in combination with HIV-1 showed no significant change in p62/SQSTM1 expression levels after days 1, 3, and 5 posttreatment compared to control (Fig. 1C and D). Together, these results show that acute HIV-1 infection induces autophagy, probably without protein degradation by lysosomes, while concurrent exposure with morphine decreases the autophagy proteins Beclin1 and ATG5, suggesting that morphine might influence the induction of autophagy.

**Effect of HIV-1 infection and morphine on dysregulation of autophagic flux in microglial cells.** An increase in LC3-II is suggestive of enhanced autophagic activity or a reduced turnover of autophagosomes due to a block in the autophagosomal maturation pathway, as previously reported (39). Our initial assessment of autophagic flux in microglia examining total p62/SQSTM1 levels showed no apparent changes after HIV-1 infection and/or treatment with morphine. We therefore examined autophagic flux at the single-cell level using a tandem mRFP-GFP-tagged LC3 reporter for fluorescence analysis, and cells were imaged by confocal microscopy (Fig. 2A and B). This assay uses the differential pH stability of the green fluorescent protein (GFP) and the monomeric red fluorescent protein (mRFP) to monitor autophagic flux. GFP has a relatively higher pKa value (pKa = 6.2) and loses

fluorescence in a lysosomal acidic environment compared to mRFP (pKa = 4.8), which does not, thereby allowing labeling of the autophagic compartments both before and after fusion with lysosomes. Accordingly, once autophagic flux is increased, both yellow (mRFP and GFP) and red (mRFP only) puncta are increased, while blocking of autophagosome fusion will only increase the number of yellow puncta (39). Microglial cells transiently transfected with the mRFP-GFP-LC3 construct showed abundant numbers of both yellow and red puncta, indicative of autophagy induction and flux (Fig. 2A to C). Furthermore, infection with HIV-1 caused a significant increase in autophagosome formation, indicated by the higher numbers of yellow puncta, while a small number of red puncta were detected (Fig. 2A to C), suggesting that some of the HIV-1-induced LC3-II had moved into the later stage of degradation, representing autolysosomes (Fig. 2A to C). However, the abundant correlation between green and red signals in HIV-1-infected microglia, compared to control cells, suggested a possible defect in the maturation stage of autophagosomes to autolysosomes (Fig. 2A to C). Exposure to morphine alone caused a significant decrease in the number of cells expressing both yellow and red puncta compared to control-treated cells, suggesting a decrease in autophagosomes and in autophagic flux (Fig. 2A to C). Concurrent exposure with HIV-1 and morphine caused a significant decrease in the number of cells expressing both yellow and red puncta compared to HIV-1 infection alone, suggesting that morphine might affect the virus-induced accumulation of autophagosome formation (Fig. 2A to C). Overall, the data indicate a strong correlation between increased levels of LC3 and accumulation of autophagosomes in HIV-1-infected microglia, which supports our initial observations that viral infection blocks autophagosome maturation and lysosomal fusion, while concurrent exposure with morphine influences autophagic induction.

**Morphine–HIV-1 interactive effect on intracellular pH in microglia.** One possible mechanism through which autophagy can be blocked at a late stage is via the alteration of lysosomes. Loss of acidification and/or lysosomal proteases can impede the fusion of autophagosomes with endosomes/lysosomes. Historically, it has been shown that opiate agonists, including morphine, can elicit cytoplasmic alkalinization (40, 41). Likewise, more recent data have also shown that exposure to HIV-1 Tat causes increased cellular pH, leading to inhibition of autophagy (42). Therefore, relative pH changes within individual lysosomes were monitored semiquantitatively using the pH-sensitive fluorescent dye, AO, and the formation of punctate staining was monitored based on visual assessment (Fig. 3A), as well as quantification using flow cytometry (Fig. 3D and E). AO is a fluorescent weak base that accumulates in acidic compartments and emits a red fluorescent signal that can be used to visualize lysosomes and evaluate gross abnormalities in lysosomal pH. To tease out the effect of individual viral proteins versus the effect of whole virions, the HIV-1 proteins Tat and gp120 were used since these proteins are known to be secreted by infected cells, are neurotoxic and, most importantly, are known to have an interactive effect with morphine (5). Microglia were treated with gp120 and Tat alone or in combination with morphine, as well as the lysosomal fusion inhibitor, chloroquine, which was used as a negative indicator of acidic vesicular formation. After 8 h of treatment, control cells primarily displayed green fluorescent labeling in the cytoplasmic and nuclear compartments with red fluorescence, indicating basal levels

of acidic vesicular organelles (AVO) (Fig. 3A). As expected, chloroquine-treated cells prevented dye retention in lysosomes and displayed a decrease in red signal (data not shown). Exposure to HIV-1 Tat caused a slight reduction in the number and size of acidic organelles compared to the control, while cells treated with gp120 showed an increase in the formation of AVO, as indicated by the increased number of cells expressing intense red signals, compared to controls (Fig. 3A). Exposure with morphine alone revealed a decrease in red fluorescence intensity, indicating a reduction of AVO compared to the control, and in combination with Tat caused a further decrease in acidic compartments compared to Tat treatment alone (Fig. 3A). Co-exposure with morphine and gp120 caused a minimal effect on the number and size of AVO compared to gp120 treatment alone and caused noticeable changes to cell morphology, indicated by actively blebbing cells, which are characteristic of an apoptotic phenomenon (Fig. 3A, arrows). The data suggest that morphine and viral proteins can reduce the induction of AVO formation in microglia.

Next, we examined the absolute intracellular pH of microglia quantitatively using the intracellular pH (pHi) indicator, pHrodo (Fig. 3B and C). Over the 8-h treatment period, control (medium)-treated microglia exhibited a basal pHi of ~6.57. Interestingly, when cells were treated with HIV-1 Tat, the basal pHi increased to ~7.32, while exposure with gp120 had a minimal effect on the intracellular pH levels of microglial cells. Exposure to morphine caused a significant alkaline shift in pH to ~7.71 compared to the pH of control-treated cells (6.57), while concurrent exposure with Tat caused a further shift to ~7.88 compared to the pH of Tat treatment alone (7.32), and a significant increase compared to control-treated cells (Fig. 3B). After a 24-h treatment period in conditioned medium, basal pHi levels in microglial cells were decreased to ~5.59, while pHi levels in cells treated with Tat and morphine remained elevated compared to gp120 and were significantly elevated compared to control-treated cells (Fig. 3C). The overall findings using AO staining correlate with the pH levels detected with pHrodo and suggest that morphine and viral proteins can cause a shift in intracellular pH that leads to decreased AVO formation in microglia.

We then verified our data with individual viral proteins by using HIV-1$_{SF162}$-infected microglial cells alone or in combination with morphine treatment. After 24 h of treatment, acidic vesicles stained with AO were quantified by flow cytometry analysis to detect the red and green fluorescent labeling (Fig. 3D and E). Control-treated cells expressed ca. 50% acidic compartments, while HIV-1 infection caused a significant increase in acidic vesicles (to ca. 65%), compared to control cells alone. Exposure to morphine alone caused a decrease in AVO-positive cells compared to control cells and in combination with HIV-1 caused a decrease in virus-induced AVO-positive cells compared to HIV-1-infected cells and noninfected control cells (Fig. 3D and E). Overall, the combined findings suggest that acidic pH is required for AVO formation in microglia, and alkalization by morphine alone or in combination with HIV-1 Tat can impair autophagosomal-lysosomal fusion.

**Autophagy is important for HIV-1 replication and displays nonspecific interactions with morphine in microglia.** Since HIV-1 infection induces autophagy in microglia, we determined the function of the host autophagic pathway on viral replication. Opioids, including morphine, induce a number of immuno-
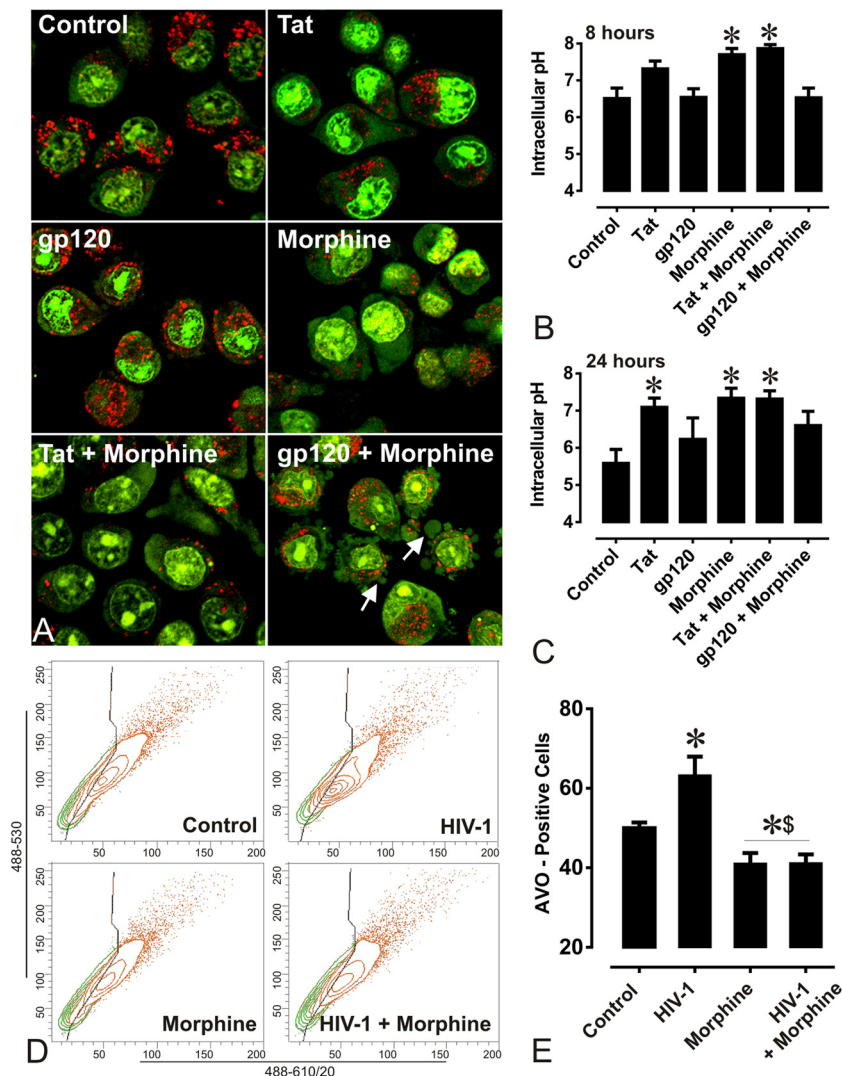
**FIG 3** HIV-1 protein, virion, and morphine effects on the number of acidic vesicles and pH levels in microglia. (A) Representative fluorescently labeled microglia with the indicated treatments were examined using confocal microscopy at ×63 magnification following acridine orange (AO) staining. Under acidic conditions, AO-stained cells fluoresce bright red, whereas basic environments such as the cytoplasm and nucleolus fluoresce bright green and dim red. Arrows indicate actively blebbing cells. Determination of intracellular pH values at 8 h (B) and 24 h (C) posttreatment using pHrodo. Values were determined from standard curves and are given as the means ± the SEM of three independent experiments. One-way ANOVA, followed by Bonferroni's *post hoc* test for multiple comparisons, was used to assess statistical differences (*, $P < 0.05$ versus control). (D) Acidic vesicular organelles (AVO) were analyzed and quantified by flow cytometry. Representative histograms of AO-stained microglia with the indicated treatments, displaying the *x* and *y* axes as increasing green and red fluorescence intensities, respectively, are shown. (E) Graphical representation of the percentage of acidic vesicles from panel D. Error bars show the SEM of three independent experiments. One-way ANOVA, followed by Bonferroni's *post hoc* test for multiple comparisons, was used to assess statistical differences (*, $P < 0.05$ versus control; $, $P < 0.05$ versus HIV-1).

modulatory effects leading to enhanced replication of HIV-1 in numerous immune cell types (26). We therefore tested whether autophagy influences the viral replication cycle in this cell type and whether coexposure with morphine converges at this pathway to enhance viral replication. RNA interference (RNAi) was directed against the autophagy gene *BECN1* (*ATG6*), which is required for the induction of autophagy (18, 36, 43). The levels of p24$^{gag}$ antigen were examined in the supernatant from HIV-1$_{SF162}$-infected microglial cells by ELISA and showed that morphine significantly enhanced viral replication in control siRNA-treated cells as expected (Fig. 4A). However, silencing of Beclin1 caused a significant decrease in virus titer compared to viral rep-

lication in control siRNA-treated cells, while coexposure with morphine caused a significant increase of ~1.3-fold in HIV-1 replication, despite the inhibition of Beclin1 (Fig. 4A). A cell viability assay was performed on cells at 48 h posttreatment with siBeclin1, and a small cell growth reduction of <10% was observed for cells transfected with Beclin1 siRNA (Fig. 4B), showing that the decreased viral replication was not likely due to cellular toxicity. The silencing efficiency of Beclin1, as determined by Western blotting, showed an inhibition of expression between 60 and 70%, and Beclin1 silencing downregulated the induction of autophagy, as detected by a reduction in LC3-II lipidation (Fig. 4C). Overall, our data are in agreement with others showing that (i) morphine en-
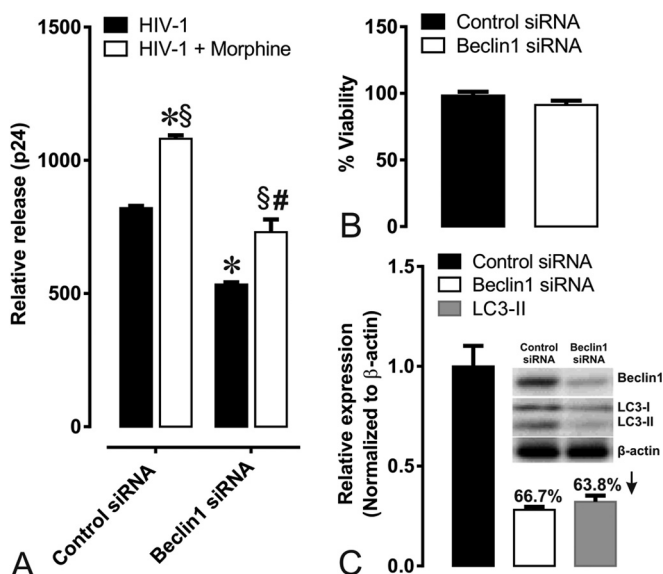
**FIG 4** siRNA against Beclin1 inhibits HIV-1 replication but not morphine-enhanced viral replication in microglia. (A) HIV-1 replication was detected in human microglia transfected with Beclin1 siRNA or control siRNA with or without morphine treatment. HIV-1 p24$^{gag}$ protein levels were monitored by ELISA. Values were determined from standard curves and are given as means ± the SEM of three independent experiments. One-way ANOVA, followed by Bonferroni's *post hoc* test for multiple comparisons, was used to assess statistical differences (*, $P < 0.05$ versus HIV-1 [siControl]; #, $P < 0.05$ versus HIV-1 + morphine [siControl]; §, $P < 0.05$ versus HIV-1 [siBeclin1]). (B) A cell viability assay was performed 48 h after transfection with siBeclin1 and siControl. The data are the percentages of viable cells ± the SEM from three independent experiments. (C) Beclin1 silencing efficiency was measured by Western blotting. Representative Western blot image and graphical representation showing Beclin1 and LC3 expression normalized to β-actin. The level measured in the control siRNA cells was set to a value of 1. Error bars show the SEM from three independent experiments.

hances HIV-1 replication (26) and (ii) that the autophagic pathway and its regulatory components contribute to HIV-1 replication in macrophages (23, 44, 45). Furthermore, the results demonstrate a Beclin1-independent pathway through which morphine enhances HIV-1 replication in microglia.

**Autophagy is important for HIV-1-induced inflammatory responses and displays nonspecific interactions with morphine in microglia.** Inflammation plays an important role in the pathogenesis of HIV-1, and elevated levels of TNF-α, IL-6, IL-8, RANTES, and MCP-1 are detected in HIV-1 encephalitic brains (46–48). Since we observed that autophagy functions in regulating viral replication in microglia, we next examined the role of autophagy in regulating the expression of the above-mentioned proinflammatory cytokines and chemokines in supernatants from HIV-1-infected microglia exposed to morphine. As expected, acute infection with HIV-1$_{SF162}$ induced a significant increase in the release of the chemokines IL-8, MCP-1, and RANTES compared to control cells (Fig. 5A to C). However, exposure with morphine alone showed a minimal effect on chemokine secretion compared to control-treated cells, while concurrent exposure with HIV-1 infection caused a significant increase in HIV-1-induced IL-8 release compared to HIV-1 infection alone (Fig. 5A). Furthermore, secretion of the cytokines IL-6 and TNF-α were found to be exclusively associated with the HIV-1 inflammatory system and exposure to morphine alone or in combination with

HIV-1 had no effects on their production (Fig. 5D and E), while the secretion of IL-1 was not induced by either stimuli (Fig. 5F). Blockade of the autophagy pathway with siBeclin1 markedly reduced TNF-α release compared to control cells (Fig. 5E), but, more importantly, siBeclin1 inhibited HIV-1-induced TNF-α, MCP-1, and RANTES release (Fig. 5B, C, and E) and showed no effect on IL-6 and IL-1 secretion (Fig. 5 D and F). In contrast, blocking the autophagy pathway with siBeclin1 did not affect the morphine/HIV-1-induced IL-8 response. Overall, the data suggest that autophagy mediates inflammation in microglia and can function as a negative regulator of the HIV-1-induced release of inflammatory molecules, whereas morphine uses a Beclin1-independent mechanism to enhance HIV-1 pathogenesis.

## DISCUSSION

Despite the success of antiretroviral therapy in reducing virus titers in blood plasma to nondetectable levels, neuronal damage and neurological deficits persist from HIV infection because these medications do not target inflammation. Therefore, in order to prevent pathological damage to the brain during HIV infection, it is of utmost importance to control microgliosis. Although opiate drug abuse and infection with HIV-1 are interlinked epidemics (3, 4) and can exacerbate the neuropathogenesis of HIV-1 infection and neuroAIDS (5), the mechanism underlying microglial activation in HIV-1 infection in the context of opioid abuse remains unclear. Here, we show that acute HIV-1 infection induces autophagy and at the same time causes an accumulation of lipidated LC3 and autophagosome formation by examining LC3-II protein levels by Western blotting and direct visualization of autophagic flux with the mRFP-GFP-LC3 reporter by confocal microscopy. The mRFP-GFP-LC3 construct was used since p62/SQSTM1, while considered a good marker to detect autophagic flux, can be upregulated and degraded under conditions not related to the autophagy pathway (49). Our findings correlate with those of others showing induction and disruption of autophagy with HIV-1-infected cells. Depending on the cell type, HIV-1 infection can either induce or downregulate autophagy (25, 50), and in some cases HIV-1 infection can also modify autophagy in uninfected bystander cells (44, 51). We further showed that concurrent exposure with morphine caused a significant decrease in autophagosome maturation (Fig. 2) and a significant decrease in AVO formation (Fig. 3). Loss of acidification and/or lysosomal proteases can impede the fusion of autophagosomes with lysosomes, which can also impair autophagy. Using the cationic dye AO and the intracellular pH detector, pHrodo, we showed that exposure to morphine and HIV-1 Tat creates a basic cellular environment. Although the detection of AVO is not a direct measure of autolysosomes, increased AVO correlates with increased autophagosomes, indicating the formation of autolysosomes. Therefore, the combined alkalization by morphine and Tat could impair autophagosomal-lysosomal fusion, which would account for a mechanism contributing to the suppression of autophagic flux with concurrent exposure (Fig. 2). Numerous pathogens can counteract the beneficial functions of autophagy in order to halt the process to their advantage (52–55) and enhance their replication (56) or to release new progeny virions (57, 58). Concurrent exposure with morphine significantly decreased HIV-1-induced Beclin1 expression, although increased LC3 expression was still detected (Fig. 6). Although we do not know the exact reason for this discrepancy, we speculate that it could be related to active viral
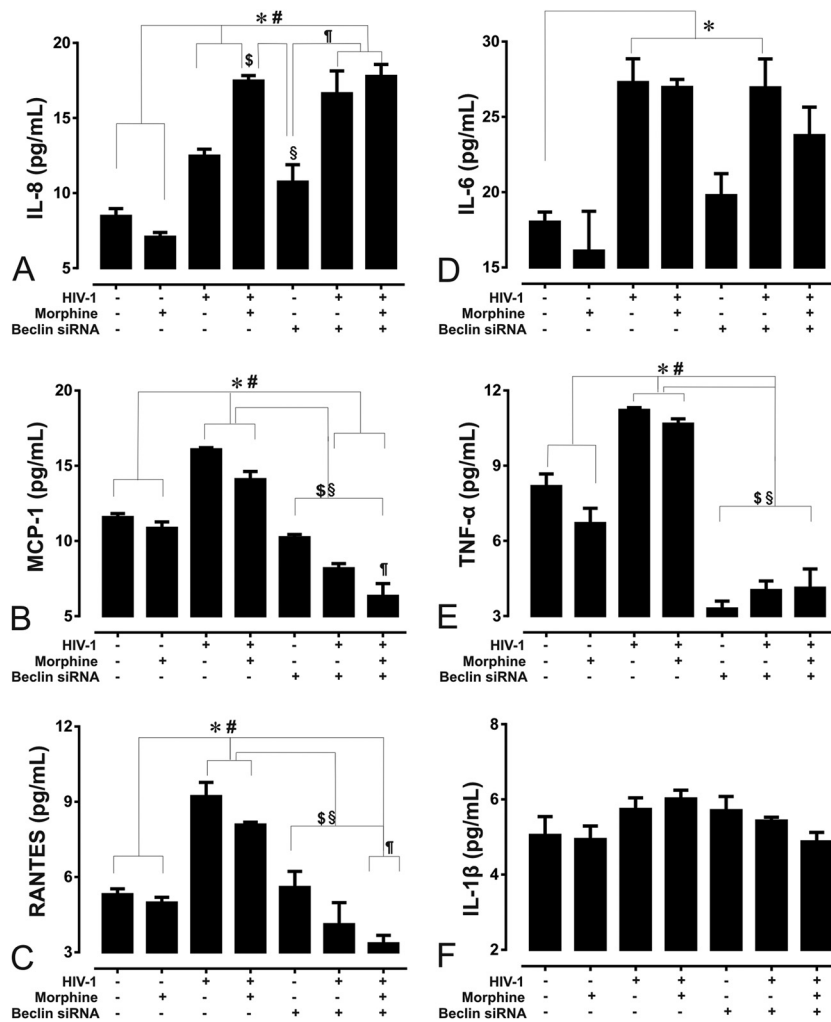
**FIG 5** siRNA against Beclin1 inhibits the HIV-1-induced, but not the interactive morphine-HIV-1 inflammatory response in microglia. Cell culture supernatants from human microglia transfected with siBeclin1 or siControl and treated with HIV-1 and morphine as indicated were used to detect the chemokines IL-8 (A), MCP-1 (B), and RANTES (C) and the cytokines IL-6 (D), TNF-$\alpha$ (E), and IL-1 (F) by ELISA. Values were determined from standard curves and are given as means $\pm$ the SEM of three independent experiments. One-way ANOVA, followed by Bonferroni's *post hoc* test for multiple comparisons, was used to assess statistical differences (*, $P < 0.05$ versus control; $, $P < 0.05$ versus HIV-1; #, $P < 0.05$ versus morphine; §, $P < 0.05$ versus HIV-1 + morphine; ¶, $P < 0.05$ versus siBeclin1).

replication. One interesting study by Kyei et al. showed that the viral protein Nef can block Gag-induced autophagosome maturation into autolysosomes via the interaction with Beclin1, leading to an accumulation of autophagosome formation and the enhancement of viral replication (45). Accordingly, a decrease in Beclin1 expression with concurrent morphine exposure could obscure the interaction between HIV-Nef and Beclin1, leading to a decrease in HIV-1-induced autophagosome formation with morphine exposure. Beclin1 levels were significantly reduced in microglia infected with HIV-1 receiving morphine treatment compared to those with HIV-1 infection alone, and this was apparent at both days 1 and 3. However, at day 5 the protein levels of Beclin1 were reduced to basal levels, and there was no significant difference between the treatments. The reduction in Beclin1 levels at day 5 can be explained by the likelihood that autophagy in HIV-1-infected and morphine-treated microglia is transiently induced, suggesting that autophagy is required for initial viral replication and that once high virus titer is established, HIV-1 no longer uses

this pathway. Since morphine uses a Beclin1-independent mechanism to increase viral replication (Fig. 6), the accumulation of autophagosome formation is not critical for the enhancement of viral replication in the context of opioid abuse. Further studies are needed to delineate the relationship between autophagosome formation and HIV-1 replication with morphine in microglia.

Studies have shown that the induction of autophagy is necessary for the biosynthesis, processing, and/or assembly of HIV-1 intermediates (23–25), while it has also been demonstrated that the later stages of autophagy (i.e., lysosome fusion and proteolysis) can reduce HIV-1 virion production (45). Using silencing of Beclin1, which is a more generally robust approach to result in complete inhibition of macroautophagy, we showed that autophagy is an essential mechanism through which HIV-1 replication is mediated, while enhanced replication of HIV-1 with concurrent exposure to morphine does not necessarily converge at this point (Fig. 6). In addition, a close relationship between inflammation and autophagy has been shown in a plethora of dis-
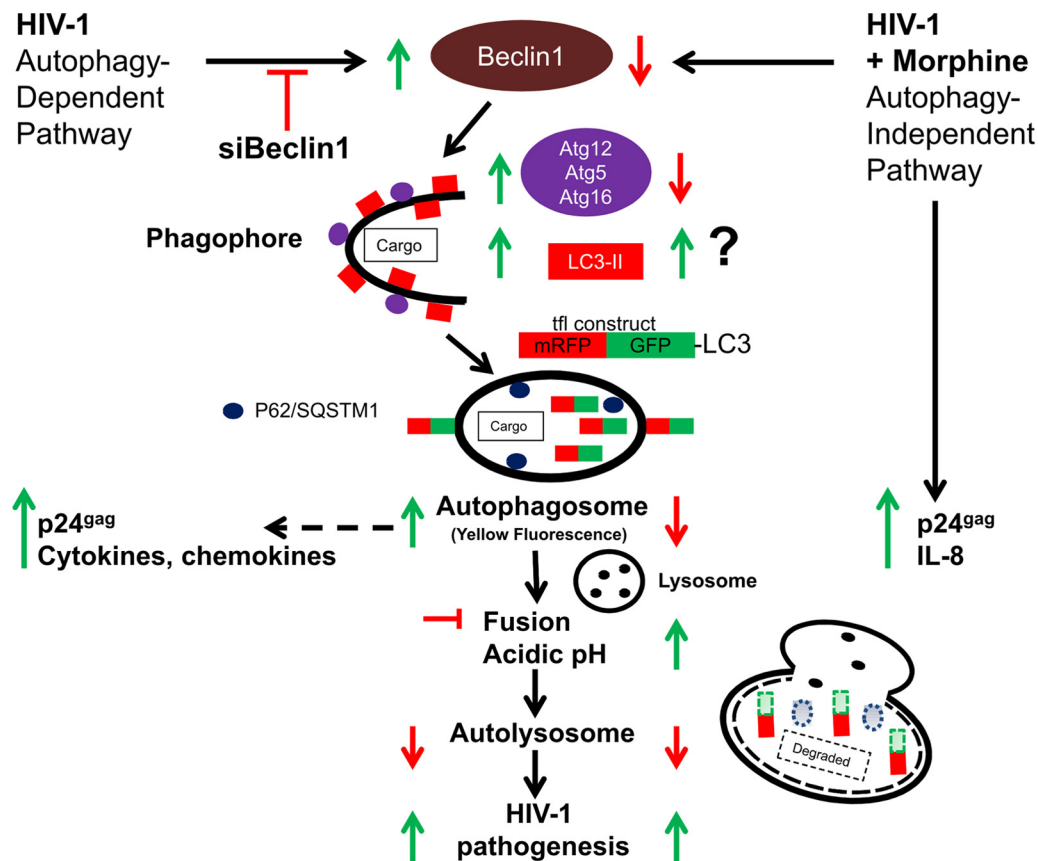
**FIG 6** Proposed model for the role of autophagy in HIV-1 replication and its biological function in microglial immune responses in the context of opioid abuse. Green arrows indicate an induction and red arrows indicate a reduction of protein expression. "⊣" indicates protein inhibition. HIV-1 infection blocks autophagosome maturation and induces an accumulation of autophagosomes, leading to the enhancement of viral replication and increased virus-induced inflammation (dotted arrow). Concurrent exposure to morphine decreases Beclin1 expression and autophagosome accumulation and still leads to induced viral replication and inflammation, suggesting that the accumulation of autophagosome formation is not critical for the enhancement of viral replication in the context of opioid abuse. How LC3-II is induced with concurrent exposure to morphine is unknown (?) in the present study. The result suggests that the increased pathogenesis of HIV-1 mediated by autophagy is exacerbated by opiate abuse via an indirect mechanism.

eases, including pancreatic and liver cancers, diabetes, cystic fibrosis, and the different manifestations of inflammatory bowel diseases (13, 59–61). More relevant to the present study, recent evidence has shown a strong association between deficits in autophagy and HIV-1-associated dementia (HAD), using postmortem brains from HIV-1-infected individuals and SIV-infected monkeys (62–65). Since inflammation plays an important role in the pathogenesis of HIV-1 and elevated levels of TNF-α, IL-6, IL-8, RANTES, and MCP-1 are detected in HIV-1 encephalitic brains (46–48, 66), our study examined these inflammatory molecules. Silencing of Beclin1 was also used to determine the importance of autophagy in mediating HIV-1-induced inflammatory responses and showed that the limited interactive effect of morphine on HIV-1-induced inflammation does not necessarily converge at this point in microglia. Although others have shown that silencing of Beclin1 can slow cell growth (24), potentially reducing HIV-1 replication and inflammation in an autophagy-independent manner, based on the cell viability assay which showed minimal cell death it can be concluded that the decreased viral replication and inflammatory responses we observed were probably not due to cellular toxicity. Exposure to morphine alone had a minimal effect on cytokine release, while in combination with

HIV-1 significantly augmented the HIV-1-induced IL-8 response. Healthy individuals have low detectable levels of IL-8, but its expression can be rapidly induced by phagocytes and mesenchymal cells in response to proinflammatory cytokines such as TNF-α or IL-1, as well as to viral products and cellular stress (67). IL-8 activates neutrophils inducing chemotaxis, exocytosis, and the respiratory burst and, more importantly, studies have shown that IL-8 stimulates HIV-1 replication in macrophages and T lymphocytes and that increased levels of IL-8 are present in the lymphoid tissue of patients with AIDS (68). However, the interactive effects of morphine on HIV-1 replication and HIV-1-induced IL-8 release were not altered by a blockade in the autophagic process.

In conclusion, the importance of autophagy in HIV-1 replication and the biological consequences of infection in microglial immune responses in the context of opioid abuse has been a largely unexplored area. To determine whether opiates converge at the level of the autophagy pathway to augment the neuropathogenesis of HIV-1, we showed a direct role of autophagy in viral replication and inflammatory host responses, while the combined effects of morphine on these processes use an indirect mechanism that did not require the protein Beclin1. Although Beclin1 expression levels were upregulated by HIV-1

infection and morphine alone at days 1 and 3 posttreatment, in combination of HIV-1 infection and morphine, Beclin1 protein levels were significantly reduced to near control levels (Fig. 1), suggesting that increased expression of this protein is not required for morphine-induced viral replication and inflammatory responses. In addition, silencing of the Beclin1 gene (*BECN1*) showed minimal reversal on the effect of morphine-enhanced cytotoxicity, suggesting that its presence is not required for morphine-induced viral replication and inflammatory responses. Although we do not know the exact mechanism through which morphine enhanced HIV-1-mediated cytotoxicity, we and others have previously shown that morphine in combination with HIV-1 viral proteins significantly induces calcium release from the endoplasmic reticulum (ER) or extracellular spaces of astrocytes and that the enhanced cytotoxicity of HIV-1 viral proteins by opiates in astrocytes is mediated through a calcium-dependent pathway (6, 7, 69). In addition, studies by others have shown that increased calcium release in the cytosol from the ER due to ER stress can activate various kinases and proteases possibly involved in autophagy signaling (70–72), and while this is speculative, the induced calcium release by morphine and HIV-1 could promote autophagy using a calcium-dependent pathway. Although Beclin1 is regarded as an essential protein in the activation of the autophagic pathway, numerous studies have shown induction of a noncanonical Beclin1-independent autophagy (73–75). Thus, while our studies have shown that Beclin1 is not necessary for morphine-induced pathogenesis in HIV-1-infected microglia, we cannot rule out the possibility that autophagy may be involved in morphine-enhanced cytotoxicity through convergent effects on calcium. Studies are being conducted in our laboratory to establish the putative role of calcium-induced autophagy as a mechanism responsible for morphine-enhanced cytotoxicity. To our knowledge, this is the first study examining the involvement of autophagy in HIV-1 replication in conjunction with opioid exposure of human microglial cells. Although the detailed relationship between autophagy, HIV-1 propagation, and opioids in microglial cell function needs to be further discerned, the results from the present study should increase the understanding of this process and should greatly improve our knowledge in the pathogenesis of HIV-1 resulting from substance abuse to provide insight for the design of candidate antiviral therapies targeting drug-abusing individuals.

## ACKNOWLEDGMENTS

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